The Effect of a Low-Fat, High Fiber, Fruit and Vegetable Intervention on Rectal Mucosal Proliferation

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BACKGROUND. Because studies of diet and colorectal carcinoma tend to be large and complex, researchers have long been interested in the investigation of dietary exposures in relation to putative intermediate markers of large bowel malignancy, such as colorectal epithelial cell proliferation. The basic hypothesis underlying these investigations is that specific dietary components may reduce or increase the rate of cell proliferation, which, in turn, may reduce or increase neoplastic changes in the large bowel.

METHODS. The authors assessed the effects of a 4-year, low-fat, high-fiber, fruit and vegetable-enriched dietary intervention on colorectal epithelial cell proliferation among 399 participants from the Polyp Prevention Trial, a randomized multicenter trial with adenoma recurrence as the primary endpoint. Rectal biopsies were taken from flat, normal appearing mucosa on patients at baseline, after 1 year, and after 4 years. Two assays, bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA), were used to evaluate two summary measures of proliferation: the labeling index (LI) and the proliferative height (PH).

RESULTS. There were no significant differences between changes in LI and PH over the 4-year period for the intervention and control groups. This finding parallels the finding in the larger primary study, in which the dietary intervention did not alter adenoma recurrence rates.

CONCLUSIONS. A low-fat, high-fiber, fruit and vegetable-enriched dietary intervention did not alter rectal mucosal cell proliferation rates. ***Cancer* 2003;98:1161–8.** *Published 2003 by the American Cancer Society.**

KEYWORDS: rectal mucosal cell proliferation, Polyp Prevention Trial, dietary intervention, adenoma recurrence, intermediate endpoint.

Because intervention and observational studies of diet and colorectal carcinoma tend to be large and complex, researchers have long been interested in the investigation of dietary exposures in relation to putative intermediate markers of large bowel malignancy, such as colorectal epithelial cell proliferation.[1] The basic hypothesis underlying these investigations is that specific dietary components may reduce or increase the rate of cell proliferation, which, in turn, may reduce or increase neoplastic changes in the large bowel. In that regard, several studies have examined colorectal epithelial cell proliferation in relation to consumption of specific micronutrients and macronutrients.[2–14]

In this article, we examine the effects of a multifactorial nutritional intervention on colorectal mucosal cell proliferation. In particular, we hypothesized that a 4-year, low-fat, high-fiber, high fruit and vegetables dietary intervention program would reduce the rate of cell proliferation measured by the overall labeling index (LI) and the mean proliferative height (PH). We tested this hypothesis by comparing

changes in proliferation indices in an intervention group and a control group at three different time intervals, from baseline to Year 1, from baseline to Year 4, and from Year 1 to Year 4.

MATERIALS AND METHODS

Patients

Biopsies were taken from 404 different individuals who participated in the Intermediate Endpoint Substudy (IES) of the Polyp Prevention Trial (PPT). The PPT, a multicenter randomized controlled trial, was designed to examine the effect of a low-fat, high-fiber, high vegetable and fruit dietary pattern on the recurrence of adenomatous polyps of the large bowel. Details of the study design, eligibility criteria, randomization procedure, dietary intervention, and endpoint assessment have been reported previously.[15,16] In summary, 2079 men and women age 35 years or older who had 1 or more histologically confirmed colorectal adenomas removed within 6 months before randomization were assigned randomly to 1 of 2 groups: an *intervention group* that received intensive counseling to achieve a diet that was low in fat (20% of total calories), high in fiber (18 g of dietary fiber per 1000 kcal), and high in fruits and vegetables (3.5 servings per 1000 kcal), and a *control group* that did not receive special diet instructions. At baseline and every year thereafter, each participant completed a 4-day food record followed by a food frequency questionnaire. In addition, unscheduled 24-hour dietary recalls were administered each year to a newly selected, random sample of 10% of participants.

Participants entered the study after undergoing complete colonoscopy and removal of adenomatous polyps and had repeat colonoscopies after 1 year and after 4 years of follow-up. The primary endpoint of the PPT was adenoma recurrence. An adenoma was defined as recurrent if it was found during any endoscopic procedure after the 1-year colonoscopy or, for participants who missed the 1-year colonoscopy, during any endoscopic procedure performed at least 2 years after randomization.

A total of 1905 randomized patients completed the study. Of the 958 patients in the intervention group and the 947 patients in the control group who completed the study, 39.7% and 39.5% of patients, respectively, had at least 1 recurrent adenoma ($P = 0.98$). The rate of recurrence of large and advanced adenomas also was similar between the two groups. Informed consent was obtained from all participants in the study. For details, see Schatzkin et al.[17]

In the IES, for which we present analyses in this article, rectal biopsies were taken on 404 of 743 eligible individuals (54.4%) at either 1, 2, or all 3 time points: baseline (T_0), after 1 year (T_1), and after 4 years (T_4) of follow-up at 3 of the 8 PPT clinical centers, the Kaiser Foundation Research Institute in California, the University of Utah in Salt Lake City, and the Walter Reed Army Medical Center in Washington, DC. There were no exclusion criteria beyond the original PPT criteria.[17] Participants were to use either no bowel preparation or a tap-water or normal saline enema following a Golytely (Braintree Laboratories, Braintree, MA), Nulytely (Braintree Laboratories), Colyte (Schwartz Pharma, Mannheim, Germany), or magnesium citrate preparation. The protocol for bowel preparation was not standardized across centers, because they were allowed the flexibility to prepare their patients in keeping with local practice. However, all patients within a given center followed the same bowel preparation procedure. For the biopsy procedures, no bowel preparation was used at Utah; at Kaiser and Walter Reed, Golytely-type bowel preparations were used. Informed consent was obtained from all participants in the substudy.

The biopsies at all time points were taken shortly before the colonoscopic examinations, and the standard was to have nothing by mouth from midnight on the night prior to the colonoscopy. The first biopsy procedure (T_0) had to be carried out within 8 weeks of randomization. The second biopsy procedure had to take place 1 year after randomization (T_1), either at the time of (and part of) the T_1 colonoscopy or as a separate proctoscopic procedure performed within 30 days prior to the T_1 colonoscopy. If biopsies could not be collected during or prior to colonoscopy, then they could be collected no earlier than 7 days after colonoscopy and within 6 months of the 1-year randomization anniversary date. Similar to the protocol for the second biopsy, the third biopsy procedure had to take place 4 years after randomization (T_4), either at the time of (and part of) the T_1 colonoscopy or as a separate proctoscopic procedure performed within 30 days prior to the T_4 colonoscopy. If biopsies could not be collected during or prior to colonoscopy, then they could be collected no earlier than 7 days after colonoscopy and within 3 months of the 4-year randomization anniversary date.

Cell Proliferation Assays

The protocol called for eight pinch biopsies to be obtained from each participant at each visit at the same time using standard forceps. Each biopsy was to be taken 8–10 cm from the anal verge. The biopsy location was noted during the procedure. Three biopsies were analyzed with the bromodeoxyuridine assay (BrdU) assay, three biopsies were analyzed with the proliferating cell nuclear antigen (PCNA) assay, and

two biopsies were quick frozen. The biopsies were removed from the endoscopy forceps and immediately placed on a strip of bibulous paper and immersed in minimal essential medium (Sigma Chemical Company, St. Louis, MO). Within 15 minutes, the biopsies were oriented in the paper strip to maximize exposure to the medium containing BrdU or fixative, respectively. For the BrdU assays, the paper strips with the biopsies were placed in disposable borosilicate sample vials with minimal essential medium containing 50 μM BrdU (Sigma Chemical Company), and 2 mL of 95% O_2/5%CO_2 were injected into the tube. The biopsies were then incubated for 1 hour at 37 °C with agitation. After the incubation, the medium was gently removed from the sample vial, and the vial was then refilled with 70% ethanol. For the PCNA assays, the minimal essential medium was removed after the orientation procedure, and the shipping tube was filled with 70% ethanol. For both types of assays, the biopsies were batched and put in a central repository before they were shipped to The University of Texas M. D. Anderson Cancer Center (M. D. Anderson) for analysis and storage. At M. D. Anderson, the biopsies were processed for histologic sectioning and embedded in paraffin. Sections (4 μm thick) were cut from the samples and placed on poly-L-lysine-coated slides. Those sections of a biopsy with well oriented crypts were immunostained using an anti-BrdU monoclonal antibody (Becton-Dickinson) or an anti-PCNA PC-10 clone (Signet Laboratories Inc., Dedham, MA). Exposure to the monoclonal antibodies was assisted by the use of a semiautomated Sequenza device (Scimetric Inc., Missouri City, TX). Visualization of the labeled cells was achieved by using the immunoperoxidase method with diaminobenzidine as the chromogen.

Scoring the Biopsies

Each biopsy was scored independently by two or three different scorers out of a pool of five at M. D. Anderson. For each biopsy, the scorer first determined whether a particular crypt was well oriented and, thus, scorable. A scorable crypt was defined as one in which the base touched the muscularis mucosa and had an open lumen at the top. The scorer then counted the number and location of labeled cells within each scorable crypt by first assigning position zero to an unlabeled crypt in the bottom center of the crypt and then counting a continuous column of cells along each of the two crypt walls. The scorers then determined which cells were labeled and which were not. If all three biopsies of a particular assay failed to yield a total of at least eight scorable crypts, then new sections were recut from the block. For instances in which one of the assays could not be evaluated, only results on the other assay were recorded. For further details on the assays and the scoring, see Kulldorff et al.[18]

Three hundred ninety-nine of 404 IES participants had at least one biopsy that could be evaluated and formed the basis of our analysis. We based our investigation on two summary measurements, the LI and the PH. The LI of a crypt was calculated by dividing the number of labeled cells in a crypt by the total number of crypt cells. We multiplied this ratio by 100 to present the LI as a percentage. The biopsy level LI was computed as the average of all crypt LI values in the specific biopsy, and the patient LI was computed as the average of all crypt LI values over all crypts and biopsies taken for the individual patient. The patient level LI was computed separately by scorer and averaged across scorers. All of the results presented in this report are based on the scorer-averaged patient level LI, which incorporated multiple counts of any crypts that were scored by multiple scorers.

The relative height of a labeled cell is the position of that cell divided by the height of the crypt. The relative heights of all labeled cells were averaged for each biopsy, as scored by a particular scorer, and over all biopsies for a patient to obtain the patient-level PH. Thus, PH indicates the degree to which the proliferative zone has extended upward within a crypt. An assessment of the variability in LI and PH, as measured by both assays, and factors that influenced that variability can be found a report by in McShane et al.[19]

Statistical Analysis

We used subscripts 0, 1, and 4 to indicate measurements at baseline, at 1 year, and at 4 years after randomization, respectively. The first analysis was based on group level data. We compared the group level mean LI_0, LI_1, and LI_4 values and PH_0, PH_1, and PH_4 values as well as the mean differences in LI_4–LI_0, LI_1–LI_0, and LI_4–LI_1 and the differences in PH_4–PH_0, PH_1–PH_0, and PH_4–PH_1 between the intervention group and the control group with an unpaired t test using two independent samples. Each difference represents a change from baseline, and the analysis of differences of differences adjusted for different distributions of baseline levels in the treatment group and the control groups.

In a second analysis, we fit linear regression models to the patient level differences in LI_4–LI_0, LI_1–LI_0, and LI_4–LI_1 and the differences in PH_4–PH_0, PH_1–PH_0, and PH_4–PH_1, including treatment group, gender, race, age, and clinical center as covariates (PROC GLM; SAS version 8.0; SAS Inc., Cary, NC). To check normality, we examined the data on both untrans-

formed and log-transformed scales using quantile plots and histograms. The differences in LI and PH as well as log LI and log PH seemed to be approximated reasonably as normally distributed.

In a third analysis, we used the patient level data (LI_0, LI_1, LI_4 and PH_0, PH_1, PH_4) to analyze the observations from the three time points simultaneously. We fit longitudinal regression models to the proliferation indices, simultaneously estimating the coefficients of visit time and diet assignment and their interaction, controlling for gender, age, race, and clinical center (PROC GENMOD; SAS version 8.0). This procedure is based on the generalized estimating equation[20]; it provides a method of taking correlations of individuals across time into account. We assumed an equicorrelated working correlation matrix (i.e., equal correlation among the observations from different time points) in those calculations, although other working correlations yielded similar results.

We also analyzed the associations between reported diet and proliferation measurements directly by fitting generalized estimating equation models to the patient level data (LI_0, LI_1, LI_4 and PH_0, PH_1, PH_4). To account for lagged effects of changes in diet, some of the models included interaction terms between visit time and dietary factors.

RESULTS

For the PCNA assay, LI and PH information was available on 399 of 404 patients in the study. Of those 399 individuals, 108 patients in the diet arm and 108 patients in the control arm had measurements for all 3 time points, 84 patients in the intervention arm and 74 patients in the control arm had measurements taken at 2 time points, and 13 patients in the controls arm and 12 patients in the diet arm had LI and PH information for only 1 of the 3 time points. The total number of LI and PH measurements based on PCNA for the control and intervention groups, respectively, were 155 and 159 for T_0, 166 and 172 for T_1, and 155 and 157 for T_4.

For BrdU, proliferation measurements could be evaluated on 371 patients. Information on LI and PH at all 3 time points was available for 39 patients in the control arm and 43 patients in the diet arm. A total of 194 participants (101 patients in the diet arm and 93 patients in the control arm) had LI and PH measurements for 2 of the 3 time points, and 95 participants (53 patients in the diet arm and 42 patients in the control arm) had measurements at only 1 time point. The total number of LI and PH measurements based on BrdU for the control and intervention groups, respectively, were 99 and 115 for T_0, 127 and 142 for T_1, and 104 and 103 for T_4. The sample sizes for complete information based on the BrdU assay were smaller compared with the sample sizes for the PCNA assay, because the BrdU assay was more difficult to perform and more frequently could not be evaluated.[18]

TABLE 1
Characteristics of Participants in the Intermediate Endpoint Substudy at Baseline

Characteristic[a]	Intervention group (n = 204 participants)	Control group (n = 195 participants)
Male gender (%)	67.65	68.72
Age (yrs)	61.55 ± 10.21	62.24 ± 9.85
Married (%)	79.41	84.10
Minority ethnic group (%)	19.61	15.90
More than a high school education (%)	71.08	79.49
Current smoker (%)	14.71	9.74
Vigorous or moderate activity or both (hours/week)	14.31 ± 14.62	13.85 ± 11.99
Alcohol intake (g/day)	8.23 ± 13.69	8.73 ± 15.23
NSAIDS (mg/day)	120.9 ± 339.9	191.4 ± 549.5
Body mass index (kg/m^2)	27.59 ± 3.97	27.95 ± 3.90
Current aspirin use (%)	24.02	23.08
Use of calcium supplement (%)	18.14	16.41
Use of vitamin E supplement (%)	23.04	17.95
Plasma total cholesterol (mg/dL)	198.77 ± 37.85	195.35 ± 35.44
Total serum carotenoids (μg/dL)	93.33 ± 41.43	98.45 ± 31.00
Family history of colorectal carcinoma (%)	27.45	25.13
Adenoma measuring ≥ 1 cm in greatest dimension (%)	25.98	32.82
Two or more adenomas (%)	34.31	41.03
One or more villous or tubulovillous adenomas (%)	17.16	21.54
Advanced adenoma (%)[b]	33.33	39.49

NSAIDS: nonsteroidal antiinflammatory drugs.

[a] For continuous variables, reported values are the means ± standard deviation. Percentages are reported for binary variables.

[b] Advanced adenoma was defined as adenoma measuring ≥ 1 cm in greatest dimension with at least 25% villous elements or with evidence of high-grade dysplasia (including carcinoma).

The dietary data as well as demographic, clinical, and behavioral characteristics of patients with missing data were similar to those with complete data. We based our analyses on all patients with measurements at the respective time points. To assess the possibility of informative drop-out, we also repeated all analyses for patients with complete measurements (n = 216 patients for the PCNA assay; n = 82 patients for the BrdU assay) but did not obtain significantly different estimates of regression coefficients or overall means.

Table 1 provides some basic demographic, clinical, and behavioral characteristics of the 399 participants in the IES at baseline for the intervention and control groups. Table 2 shows the dietary characteristics of the IES control and intervention groups for all three time points. There were no statistically signifi-

TABLE 2
Reported Daily Dietary and Supplement Intakes, Biomarkers, and Weight of Participants in the Intermediate Endpoint Substudy

	Intervention group			Control group		
Variable[a]	T_0 (n = 204)	T_1 (n = 202)	T_4 (n = 199)	T_0 (n = 195)	T_1 (n = 194)	T_4 (n = 184)
Fat (% of calories)	35.60 ± 7.00	35.95 ± 7.48	23.60 ± 6.86	35.95 ± 7.48	34.28 ± 7.12	33.76 ± 7.52
Fiber (g/1000 calories)	10.43 ± 5.01	17.44 ± 6.44	17.20 ± 5.91	9.74 ± 3.76	10.55 ± 3.93	10.48 ± 3.80
Fruits/vegetables (servings/1000 calories)	2.11 ± 1.02	3.35 ± 1.23 3	3.44 ± 1.31	2.02 ± 0.95	2.22 ± 0.97	2.32 ± 1.15
Calories (kcal/day)	1956 ± 536	1834 ± 495	1873 ± 496	2063 ± 665	1935 ± 665	1914 ± 536
Red and processed meat (g/day)	93.54 ± 49.29	72.23 ± 40.78	73.88 ± 41.00	98.67 ± 53.06	91.45 ± 45.39	95.57 ± 53.75
Ratio of red meat to chicken and fish	2.4 ± 2.8	1.8 ± 2.0	1.8 ± 2.2	2.3 ± 2.4	2.6 ± 4.2	2.9 ± 4.8
Whole grains (g/day)	82.33 ± 59.06	122.40 ± 76.74	121.86 ± 76.91	86.05 ± 59.49	85.73 ± 59.54	78.30 ± 57.69
Legumes (g/day)	14.3 ± 19.8	44.3 ± 41.3	46.6 ± 53.4	14.7 ± 16.9	14.6 ± 18.2	14.9 ± 16.8
Calcium from food and supplements (mb/day)	993 ± 5125	1089 ± 567	1188 ± 660	1033 ± 544	1078 ± 653	1130 ± 695
Plasma total cholesterol						
Mean ± SD (mg/dL)	198.22 ± 37.41	196.26 ± 36.05	197.49 ± 34.37	195.34 ± 35.43	197.50 ± 33.99	189.91 ± 36.23
No. of patients	93	92	85	89	88	78
Serum total carotenoids						
Mean ± SD (mg/dL)	93.29 ± 40.76	113.46 ± 60.07	105.15 ± 47.61	98.44 ± 45.22	101.95 ± 41.41	92.76 ± 7.10
No. of patients	93	93	85	89	89	78
Weight (pounds)	178.9 ± 31.9	174.2 ± 31.6	177.9 ± 33.5	183.0 ± 33.3	183.5 ± 33.8	184.5 ± 36.3

T_0: baseline; T_1: after 1 year; T_4: after 4 years; SD: standard deviation.
[a] For continuous variables, reported values are the means ± standard deviation. To convert values for cholesterol (carotenoids) to millimoles per liter, multiply by 0.02586 (0.0185). Cholesterol and carotenoids were measured after an overnight fast.

cant differences between the two groups for any of the variables presented in Table 1. The dietary, clinical, demographic, and behavioral characteristics and the changes over the 4-year study period of the participants in the IES substudy for the intervention and control groups were similar to those in the respective groups of the PPT.

Results for Changes in LI

At baseline (T_0), the mean LI values for all participants were 4.02 (n = 99 patients) and 4.29 (n = 155 patients) for the BrdU and PCNA assays, respectively, in the control group and 3.91 (n = 115 patients) and 4.16 (n = 159 patients), respectively, in the intervention group. There were no differences in the LI at baseline for the intervention and the control groups either for BrdU or for PCNA, as expected from randomization.

To determine whether there were different changes in the rates of cell proliferation in the patients assigned to the intervention arm compared with patients assigned to the control arm, we compared the differences LI_4–LI_0, LI_4–LI_1, and LI_1–LI_0 for the intervention group and the control group. If individuals who were assigned to the intervention arm had a lower rate of cell proliferation measured by LI, then we would expect to see a significant positive difference between the changes for the control group minus the changes for the intervention group. However, there is no statistically significant evidence that diet alters changes in LI more than control treatment, based on unpaired t tests, with all P values > 0.48, for differences in LI_1–LI_0, LI_4–LI_0, and LI_4–LI_1. Table 3 shows the differences in mean values as well as the associated P values.

TABLE 3
Differences in Labeling Index Scores for Control and Intervention (Diet) Arms

			Control and intervention groups		
Assay/period	Control group	Intervention group	Mean	95% CI	P value
PCNA					
T_1–T_0	0.22	0.27	−0.05	−1.68, 1.58	0.48
T_4–T_0	0.40	0.36	0.04	−1.53, 1.61)	0.50
T_4–T_1	0.14	0.09	0.05	−1.91, 2.01	0.52
BrdU					
T_1–T_0	−0.13	0.25	−0.38	−2.34, 1.58	0.35
T_4–T_0	0.33	0.46	−0.13	−2.09, 1.83	0.45
T_4–T_1	0.46	0.21	0.25	−1.71, 2.21	0.60

95% CI: 95% confidence interval; PCNA: proliferating cell nuclear antigen; T_0: baseline; T_1: after 1 year; T_4: after 4 years; BrdU: bromodeoxyuridine.

To adjust for possible effects of other covariates, we regressed the changes in LI on age, gender, clinical center, and race as well as treatment group (coded 1

TABLE 4
Differences in Proliferative Height Scores for the Control and Intervention (Diet) Arms

Assay/period	Control group	Intervention group	Control and intervention groups		*P* value
			Mean	95% CI	
PCNA					
T_1–T_0	−1.06	−1.60	0.54	−0.50, 1.59	0.31
T_4–T_0	−0.37	−0.60	0.23	−0.51, 0.97	0.53
T_4–T_1	0.03	−0.12	0.15	−0.47, 0.77	0.64
BrdU					
T_1–T_0	−2.13	−3.85	1.72	−0.44, 1.87	0.12
T_4–T_0	−0.03	0.29	−0.32	−2.10, 1.44	0.72
T_4–T_1	−0.30	0.77	−1.07	−2.55, 0.41	0.16

95% CI: 95% confidence interval; PCNA: proliferating cell nuclear antigen; T_0: baseline; T_1: after 1 year; T_4: after 4 years; BrdU: bromodeoxyuridine.

for the intervention arm and 0 for the control arm). The coefficient for treatment group was not significant in any of these analyses, confirming the findings shown in Table 4. The coefficient for treatment group corresponding to the difference in LI for T_1 and T_0, as measured by the PCNA assay, was − 0.15 with a 95% confidence interval (95% CI) of − 0.70, 0.41. The treatment coefficients for LI_4–LI_1 and for LI_4–LI_0 were 0.14 (95% CI, − 0.52, 0.80) and 0.01 (95% CI, − 0.66, 0.64), respectively. None of the coefficients of the other variables in the model were statistically significant. For the BrdU assay, we obtained similar results. The treatment group coefficients were 0.11 (95% CI, − 0.76, 0.98), 0.17 (− 0.81, 1.15), and 0.28 (− 0.74, 1.30) for the differences in LI_4–LI_0, LI_4–LI_1, and LI_1–LI_0, respectively. Increased variability and slightly smaller sample sizes for the BrdU assay are reflected in wider confidence intervals. Nonetheless, none of the differences measured by either assay showed a statistically significant difference in changes in the proliferation rate between the intervention group and the control group.

The coefficients we obtained by fitting a longitudinal regression model to the log-transformed LI for the individual data were not significantly different from zero for any of the covariates in the model (data not shown). In addition, there was no interaction between visit time and group assignment. The coefficients corresponding to visit at time T_4 were always negative, indicating an increase (although it was not significant) in the proliferation index with time. In agreement with those findings, the longitudinal model that regressed the log-transformed LI for the individual data on the dietary components did not yield any statistically significant results (data not shown). In addition, there was no interaction between visit time and dietary intake. We conclude that there was no difference in the mean LI or in changes in LI with treatment based on either of the two assays.

Results for Changes in PH

The analysis of PH yielded results similar to those for the analysis for LI. The differences of the means for the control arm and the intervention arm as well as the values for the *t* tests and the associated *P* values are shown in Table 4. For the PCNA assay, the *t* test resulted in nonsignificant *P* values for PH_1–PH_0, PH_4–PH_0, and PH_4–PH_1. All of the mean values in the intervention group were negative, suggesting that the PH was greater at earlier points in the intervention trial. This effect was not seen for the PH as measured with the BrdU assay. None of the results for PH based on a comparison of the overall mean values in the control group and the intervention group reached statistical significance (Table 4). Because a plot of the differences in PH_1–PH_0 revealed a slight deviation from normal, we also performed a Wilcoxon rank-sum test that resulted in a value for the test statistic $Z = -0.44$, with a *P* value of 0.66.

For the regression analysis for PH controlling for age, gender, clinical center, and race, we obtained estimates of treatment effects of − 2.09 (95% CI, − 6.23, 2.03) for PH_1–PH_0, 1.14 (95% CI, − 2.36, 4.65) for PH_4–PH_0, and − 0.44 (95% CI, − 1.91, 1.04) for the PNCA assay. For the BrdU assay, the values obtained were − 0.94 (95% CI, − 2.98, 1.10) for PH_1–PH_0 and − 0.44 (95% CI, − 1.92, 1.03) for PH_4–PH_0. None of the other covariates reached significance. Fitting a generalized estimating equation model that contained visit time, group assignment, gender, age, clinical center, and race as covariates and interaction terms for visit time and group assignment did not yield significant results for any of the covariates, including the interaction terms (data not shown). The corresponding longitudinal model that regressed the log-transformed PH for the individual data on the dietary components, as given in Table 2, including interaction terms with visit, did not yield any statistically significant results (data not shown). In summary, rectal mucosal proliferation measured by LI and PH based on two different assays did not differ significantly between the intervention group and the control group over a period of 4 years.

DISCUSSION

We investigated the effects of a 4-year dietary intervention on 2 measures of rectal mucosal proliferation, LI and PH, in a study that included 399 partic-

ipants. Although the dietary assessment data showed that the intervention and control groups differed substantially in the intake of fat, fiber, and fruits and vegetables, we did not find any intervention-control group proliferation differences for either the PCNA assay or the BrdU assay. The study was well powered, because the sample sizes were adequate to guarantee 80% power to detect a 10% difference in LI and a 5% difference in PH between the intervention group and the control group. These findings are consistent with the results for the primary endpoint of the study, adenoma recurrence, because there was no significant difference in recurrence rates between the intervention and the control group. If we had been able to reduce total variation of the measurements by 20%, then we would have had 80% power to detect a 7% difference in LI and a 3% difference in PH between the groups.

There may be several reasons why we did not see any differences. First, measuring proliferation is technically difficult. There are many sources of variation within and between individuals. Kulldorff et al.[18] pointed out that there are significant differences between the variance components of the two assays that have to be taken into account when dealing with multiple biopsies at multiple points in time. McShane et al.[19] showed that the size of within-person variability can be nearly as great as between-person variability, making it difficult to separate signal from noise. To control for those sources of variation, we fit similar extended variance components models to the data that, in addition to clinical center, treatment group, visit, an interaction between treatment group and visit, race, age, and gender, included hour and month of biopsy, interactions for visit and scorer, scoring date, scorer, and visit. Although it was found that some of the interaction terms were of borderline significance, there were no differences in the proliferation measures by treatment group over time, and it was found that time of biopsy was not significant. Variation due to differences in bowel preparation and endoscopist were incorporated into the variability by *clinical center*, because a single endoscopist/investigator took the biopsies at each center, and bowel preparation also was standardized for each center. Because those models do not provide additional insight into the data, we do not present the results.

By averaging over crypts, biopsy, and scorer, we reduced the impact of the variance contributions from those factors. Changes in proliferation measurements could arise from systematic changes in the scoring over time. Because those trends would affect observations in the control and intervention groups equally, we eliminated the effects by comparing differences of the changes in the control group and changes in the intervention group.

Second, our study consisted of people who were older and who had one or more histologically confirmed colorectal adenoma removed prior to study enrollment. Epithelial tissue that already has experienced neoplastic events, in a sense, may be *resistant* to interventions designed to alter proliferation measures. In addition, if nutritional factors have an impact on critical events on a molecular level early in life, then dietary changes later in life may be ineffective.

Investigations of the effect of altering dietary fat, fiber, and fruit and vegetable intake—or some combination of these factors—on rectal mucosal proliferation are sparse. Stadler et al.[21] found that dietary fat given as a bolus (of corn oil) increased proliferation. Holt et al.[13,14] recently showed that supplementation with low-fat dairy products reduced proliferation, whereas Karagas et al.[22] found that greater consumption of dairy products did not alter proliferation. Because overall fat was not necessarily reduced in these dairy product studies, those findings most likely are interpreted best with respect to a possible effect for calcium supplementation rather than fat reduction. Alberts et al.[7] found that wheat bran fiber supplementation had no effect on rectal epithelial cell proliferation. We did not find statistically significant associations between any of the reported dietary factors and rectal mucosal proliferation in a longitudinal regression model for the individual proliferation measurements.

Although it has been shown that epithelial cell proliferation measurements in the rectum parallel those in the colon,[23,24] a key question in understanding the interrelation of diet, colorectal mucosal proliferation, and neoplasia is whether proliferation findings can be extrapolated to the more advanced neoplasia/carcinoma endpoints. Two types of investigations can shed light on this question. First, by incorporating proliferation markers into a polyp trial, it may be determined whether a given nutritional intervention has similar effects on both proliferation and the more advanced neoplasia/carcinoma endpoint. With the PPT, intervention group assignment was associated with neither rectal mucosal proliferation nor subsequent adenoma recurrence.[17] In contrast, in a recently reported polyp trial,[25] calcium supplementation had no impact on mucosal proliferation, even though the intervention resulted in a modest but statistically significant reduction in neoplasia (that is, adenoma recurrence). Second, it may be ascertained in the polyp trial context whether rectal mucosal pro-

liferation measures predict adenoma recurrence. In the calcium-polyp trial, the proliferative index did not predict future colorectal neoplasia, although it was associated weakly with adenoma prevalence.[26] We will examine proliferation-adenoma relations in subsequent analyses of the PPT data.

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